ISOLATION AND IDENTIFICATION OF VOLATILE COMPONENTS OF HOST EFFLUENTS ELICITING ELECTROPHYSIOLOGICAL RESPONSES IN LONE STAR TICKS

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CHAPTER I

INTRODUCTION

The discovery and identification of olfactory stimuli associated with host-seeking activity in hematophagous arthropods has received considerable attention in recent years, with emphasis directed towards its possible utilization in control programs and population density surveys. In the area of tick research, carbon dioxide (CO_2) (Nevill 1964, Miles 1968, Wilson et al. 1972, Sauer et al. 1974) and ammonia (NH_3) (El-Ziady 1958, Haggart and Davis 1979, 1980) have been postulated as being important host-produced olfactory stimuli associated with host-locating behavior. Although laboratory and field experimentation has indicated that these compounds do elicit active behavioral responses in ticks at certain concentrations, a great deal of variability and inconsistency have been observed. Results give rise to the theory that tick host-seeking behavior may be modulated by a wide range of overlapping airborne chemical stimuli. Unfortunately, the discovery and identification of other potential semiochemicals is extremely lacking. However, behavioral studies by Lees (1948), Wilkinson (1953), Dethier (1957), and Balashov (1972) have indicated that tick host-orienting behavior may be influenced by certain organic volatile components contained in host body effluents. It is therefore evident that future investigations need to be initiated to elucidate if potential tick semiochemicals are contained in these effluents.

The objective of this study was to develop an integrated research program which would systematically collect, concentrate, and separate volatile organic components from host body effluents; and isolate and identify those particular components which produce active neurological olfactory responses in lone star ticks, Amblyomma americanum (Linnaeus).

The lack of information regarding investigations of this type in entomological research dictates that a major portion of this study be devoted to instrumental design and procedural modifications. Recent advances have been made concerning the analysis of the volatile organic components found in human urinary profiles. Therefore, certain procedures and techniques will be patterned after these studies, with sheep urine serving as our effluent of choice.

CHAPTER II

REVIEW OF THE LITERATURE

Sensory Physiology and Chemoattraction

Haller's organ, located on the dorsal surface of tarsus I of the tick foreleg, was originally described by Haller (1881) who regarded the structure as an auditory organ because of its resemblance to the auditory sacs present on the antennules of various crustaceans. This function was later challenged by Batelli (1891) who suggested that Haller's organ might serve as a means of perceiving at a distance the hosts on which ticks feed. However, Batelli was merely stating a hypothesis and provided no documentation to support his claim. In 1905 LaHille became the first investigator to supply research evidence supporting the theory that Haller's organ functions as a chemoreceptive structure. In his work, LaHille found that female Boophilus annulatus (Say) would not walk across a strip of paper impregnated with a 1% solution of Sarnol. However, if these particular ticks had their first pair of legs amputated, thus being deprived of their Haller's organs, they would not hesitate in their movement across this barrier. Behavioral studies on the sensory perceptions of Argas persicus (Oken) (Hindle and Merriman 1912) later added support to LaHille's theory of Haller's organ involvement in the perception of olfactory stimuli.

Morphologically, Nuttal et al. (1908) first determined that the Haller's organs of ixodid ticks are comprised of two distinct parts,

which they referred to as the anterior pit and posterior capsule. These observations were based on light microscopial investigations, and it was not until the advent of recent techniques such as scanning electron microscopy (SEM) that a more detailed understanding of the ultrastructure and morphology of this organ became known. Axtell et al. (1971), Bruce (1971), and Foelix and Axtell (1972) utilized SEM to study the internal structures of the posterior capsule of Haller's organ in the lone star tick, Amblyomma americanum (Linnaeus). They observed the presence of several irregularly shaped non-sensory cuticular projections (pleomorphs), along with 7-8 symmetrically arranged blunt-tipped thin-walled sensilla perforated by numerous pores. These sensilla correspond morphologically in most details to the olfactory receptors found in insects (Slifer 1970).

The neural mechanisms which control and mediate arthropod behavior and olfactory perception have been studied in detail by several authors (Erickson 1963, Huber 1967, Kaissling 1971, and Balashov 1972). Balashov noted that ixodid ticks have retained a simple bineural system consisting of both sensory and motor neurons from several pair of glomerular nuclei. These nuclei lie within the ganglia of the first leg and are connected to Haller's organ. Balashov found this particular arrangement to be specific for ixodid ticks and suggested that such a system could play an integral role in the perception of odors from a considerable distance.

Investigations on tick host-seeking activity and its relationship to the perception of olfactory stimuli have been extensively documented. Behavioral studies by Lees (1948) demonstrated that the sheep tick, Ixodes ricinus (Linnaeus), was strongly attracted to sheep wool and

other animal hair at 37°C, but was repelled by a temperature of 37°C in the absence of wool and completely insensitive to wool at room temperature. Similar investigations performed by Wilkinson (1953) on the larvae of the cattle tick, Boophilus microplus (Canestrini), supported the findings of Lees. Concerning responses to odor, Wilkinson showed that ticks were greatly attracted to a test tube rubbed in mucus, saliva, and over areas of the flank and escutcheon of cattle.

Tick attraction resulting from odors being emitted from the hair or fur of host animals was also substantiated by El-Ziady (1958) in his work on Ornithodoros erraticus (Lucas), and by Merritt (1967) on behavioral studies concerning the rabbit tick, Haemaphysalis leporispalustris (Packard). Balashov (1972), working with several ixodid species, showed that ticks which were released at varying distances from a frequently used host path would orient themselves in close proximity to that path for periods as long as one month after release. He concluded that attraction to the path was due to host odors which were present even during the absence of the host itself. More recently, Dukes and Rodriguez (1976) studied the host-seeking responses of nymphal A. americanum, Dermacentor variabilis (Say), and Rhipicephalus sanguineus (Latreilla). On responses to odor, they found that all three species were significantly attracted to a methanol extract of dog hair at 37°C, with 60-70% attraction being observed in A. americanum.

Although investigations, such as those previously mentioned, have indicated that ticks are attracted to certain volatile chemical odors being emitted by a host, only limited attempts have been taken towards the discovery, identification, and utilization of these particular compounds. Evidence supporting the theory of carbon dioxide (CO_2) involve-

ment in tick host-seeking activity have been reported by Nevill (1964), Miles (1968), Wilson et al. (1972), and Sauer et al. (1974). In his work, Nevill showed that the sand tampan, Ornithodoros savignyi (Audouin), was capable of detecting a 5% concentration of CO_2 liberated at 1 liter/min while still below the sand. He therefore concluded that CO_2 in the exhaled breath of a host, ca. 4-5% in bovines and humans (Kellogg 1970), was the main factor in the host-seeking activity of the sand tampan. Garcia (1962, 1965, 1969) also utilized CO_2 to trap significant numbers of Ornithodoros coriaceus Koch, Dermacentor occidentalis Marx, Ixodes pacificus Cooley and Kohls, and Dermacentor andersoni Stiles and suggested that the frequent passage of animals along game trails could increase the amount of CO_2 in the vicinity and ultimately cause ticks to congregate near the trails.

A similar rationale was used concerning the ammonia (NH_3) gradient which exists along frequently used host paths. In laboratory experimentation, El-Ziady (1958) was able to show that adult O. erraticus were greatly attracted to 0.5 and 1.0% solutions of NH_3. These results correlate well with electrophysiological studies by Haggart and Davis (1979, 1980), who demonstrated the presence of two types of NH_3-sensitive receptors on the first tarsi of the adult brown dog tick, R. sanguineus. Based on this data, these authors suggested that NH_3 may be a primary factor in tick host-orienting behavior.

Burris (1974) utilized an olfactometer system to test candidate chemicals for attractant properties in the lone star tick, A. americanum. Investigations on 67 inorganic or organic chemicals soluble in water or methanol revealed those compounds containing sodium to be the most attractive, with statistical analysis showing certain amino acids as the

chemicals eliciting the best responses. Wood et al. (1975) performed studies on several ixodid species, including A. americanum, and suggested that these ticks may possibly utilize simple phenolic compounds as general purpose attractants. Lastly, Dukes and Rodriguez (1976) observed that 62% of A. americanum nymphs were attracted to a 0.5% solution of N-N-diethyl-meta-toluamide. However, these authors did not speculate on the importance of this particular compound or its association with tick host-seeking behavior.

The aforementioned studies indicate the extreme lack of information regarding specific olfactory components responsible for tick host-orienting activity. Although compounds such as CO_2 and NH_3 have been shown to elicit active responses in ticks at certain concentrations, a great deal of variability and inconsistency have been observed (Dukes and Rodriguez 1976, Gearhart et al. unpublished). These results suggest that tick host-seeking behavior may be modulated by a wide range of overlapping airborne chemical stimuli. This theory closely parallels work done on several hematophagous insects, which may possibly serve as a reference for future investigations concerning tick olfactory perception.

Evidence supporting the chemoattractant properties of CO_2 in hematophagous insects include studies conducted on biting midges (Nelson 1965, Whitsel and Schoeppner 1965), black flies (Fallis and Smith 1964, Snoddy and Hays 1966), horse flies (DeFoliart and Morris 1967), and mosquitoes (Van Thiel 1947, Van Thiel and Weurman 1947, Brown 1951, Brown et al. 1951, Willis and Roth 1952, Snow 1970, Bar-Zeev et al. 1977). Concerning mosquitoes, other investigators concluded that CO_2 merely acts as an activator rather than an attractant (Rudolfs 1922, Willis

1947, Laarman 1955, Kellogg and Wright 1962, Daykin et al. 1965, Khan and Maibach 1966).

Investigations have also been conducted on parts of the human body and its products in search of specific chemical attractants for mosquitoes (Brown 1958, Khan et al. 1965, Maibach et al. 1966, Skinner et al. 1965). Muller (1968) found that human blood, urine, and sweat in their natural condition were attractive to Aedes aegypti (Linnaeus). However, when these effluents were tested in an alkaline form, they were not attractive. Muller, therefore, suggested that the organic acids found in host body effluents were the main attractive factor in mosquito host-seeking activity. Other compounds found to have chemoattractant properties include lysine and alanine (Brown and Carmichael 1961), sex hormones (Roessler and Brown 1964), carbamino compounds (Brown 1966), L-lactic acid (Acree et al. 1968, Smith et al. 1970), methionine (Ikeshoji 1967, Ikeshoji et al. 1963), unspecified blood constituents (Schaerffenberg and Kupka 1953), and lysine, cadaverine and estradiol (Bos and Laarman 1975). Even though these chemicals have been shown to have some attractant characteristics to mosquitoes, nothing has proved to be as attractive as man himself (Bar-Zeev et al. 1977, Price et al. 1979).

Electrophysiology

The use of electrophysiology as an effective means of investigating the sensory physiology of arthropods has been well documented. Concerning insects, Schneider (1957) was the first investigator to use this technique to record slow and summed receptor potentials elicited by odors in his work on the silkmoth, Bombyx mori (Linnaeus). Since then,

electrophysiology has been used to study the sensory physiology of numerous insect species, including hematophagous mosquitoes (Kashin and Wakeley 1965, Kashin 1966, Lacher 1967, 1971, Kellogg 1970, Davis and Rebert 1972, Davis and Sokolove 1975, 1976, Davis 1976, 1977), tsetse flies (Rice et al. 1973, Mitchell 1976a, 1976b), and hemipterans (Bernard et al. 1970).

Extensive electrophysiological investigations involving ticks are relatively limited. Gregson (1969) utilized electrograms to monitor the feeding behavior of D. andersoni. He observed several types of neural discharge activity and associated these with different aspects of the tick's feeding pattern. Similar feeding electrograms were conducted by Sweatman and Gregson (1970) on Hyalomma aegyptium (Linnaeus) and showed the importance of temperature on the rate of engorgement. The use of electrophysiological techniques to study the contact chemoreceptors of ticks was initially conducted by Elizarov (1964a, 1965) in his work on Hyalomma asiaticum Schulze and Ixodes persulcatus Schulze. He found that only one pair of sensilla on the distal portion of tarsus I function as contact chemoreceptors in both tick species. The pulvilli surface in I. persulcatus was also found to function as a contact chemoreceptor, but similar findings could not be confirmed in H. asiaticum. More recently, Waladde and Rice (1977) recorded electrophysiological responses to solutions of sodium chloride, adenosine triphosphate, reduced glutathion, and bovine plasma from contact chemoreceptors located in the cheliceral pit of the cattle tick, B. microplus. This work was unique in that it added a sensory function to the chelicerae of ticks, appendages which were previously regarded as mere cutting structures used in host attachment. Binnington and Rice (1977) used electrophysio-

logical techniques to record a rapid increase of spike frequency in a coxal nerve of B. microplus after topical application of an acaricidal formamidine (N-(2,4-dimethylphenyl)-N-methyl formamidine). They suggested that this compound may possibly stimulate tick detachment from a host through its action on motor neuron activity.

In the area of tick olfactory perception, Zolotarev and Elizarov (1963) electrophysiologically investigated chemoreception in I. persulcatus. After implanting a microelectrode into the base of Haller's organ and subjecting the tick to a variety of commercially prepared repellents, they suggested that these chemicals affect the tick by stimulating the olfactory receptors located in Haller's organ. Experiments by Elizarov (1964a, 1964b, 1965) on H. asiaticum and I. persulcatus provided further evidence supporting Haller's organ involvement in olfactory perception. He observed that a volley of monophasic nerve impulses could be elicited 0.5 to 1.0 second after introduction of various chemical repellents (odors) into Haller's organ.

Haller's organ involvement in the perception of the reported tick sex pheromone, 2,6-dichlorophenol (2,6-DCP), has also been documented electrophysiologically. Chow (1974) recorded electro-olfactory potentials produced in response to 2,6-DCP from the posterior capsule of Haller's organ in Amblyomma maculatum Koch, and R. sanguineus and suggested that pheromone-sensitive neurons might be found at that location. These findings were later challenged by Haggart and Davis (1981) in their work on A. americanum. Using electrophysiological techniques, these authors found receptors sensitive to 2,6-DCP in the anterior pit region of Haller's organ, but were unable to demonstrate similar findings regarding the receptors contained in the posterior capsule.

Electrophysiological measurements of tick responses to host odor was first documented by Sinitsyna (1974) in his work on H. asiaticum. After subjecting Haller's organ to odors emitted from mice, mice blood, valeric acid, and human respiration, he recorded bioelectrical activity and suggested that the olfactory receptors in this organ respond selectively and qualitatively to different host odor stimuli. More recently, Holscher et al. (1980) used electrophysiological techniques to quantitatively measure neurological responses produced from regulated levels of CO_2 stimulation in A. americanum. These authors evaluated biotic and abiotic parameters involved in tick response to CO_2 and determined that sex, age, and species, as well as humidity, temperature, and ambient CO_2 concentration quantitatively influence tick perception of CO_2.

Gas Chromatography-Mass Spectrometry

The technique known as gas chromatography was originally described by Martin and Synge (1941), and has since developed into one of the most widely used analytical procedures in the physical sciences. Until recently, these analysis have mainly concerned themselves with the separation of different classes of nonvolatile compounds from complex mixtures. However, new advances in isolation and concentration techniques have since made the chromatographic analysis of volatile complexes more feasible. Pauling et al. (1971), Teranishi et al. (1972), and Robinson et al. (1973) used high-resolution techniques to quantitatively analyze volatile compounds found in human urine. Utilizing headspace collection followed by cold trapping, these authors chromatographically separated more than 200 volatile constituents on a 1000 ft x 0.03 inch inner diameter (I.D.) stainless steel open tubular column coated with silicone oil

(SF 96). In 1973b, Zlatkis et al. described an isolation technique in which headspace analysis of urine volatiles was followed by adsorption onto the porous polymer, Tenax. The Tenax was then transferred to a modified injection port, and the volatile components thermally desorbed onto the cooled precolumn of a gas chromatograph prior to separation. These authors found that Tenax was very heat stable, and provided good adsorptivity, desorptivity, and reproducibility. Later, Versino et al. (1974) utilized a similar isolation technique and obtained 90% adsorption and desorption efficiencies on Tenax for several different classes of compounds, including amines, ketones, phenols, ethers, and alkanes.

The combined usage of gas chromatography and mass spectrometry (GC-MS) for the analysis of volatile urinary constituents has been described by several authors. Zlatkis and Liebich (1971) used GC-MS techniques to separate ca. 300 compounds, 40 of which were identified by MS. Components identified included dimethyl sulfone, pyrrole, 4-heptanone, allyl isothiocyanate,several alkyl furans, ketones, and lactones. Matsumoto et al. (1973) used a modified headspace collection technique and coinjection procedure to separate and identify 42 volatile compounds of human urine by GC-MS. Of the compounds identified, 22 had never been previously referenced in urine. Most notable of these were acetaldehyde, trimethylamine, chloroform, toluene, and benzene. Further experiments by Zlatkis et al. (1973a, 1973c) demonstrated the feasibility of using Tenax isolation and concentration techniques in conjunction with GC-MS procedures. These authors showed that Tenax-filled traps could be stored up to two weeks at room temperature, or reused for multiple sampling without any appreciable loss of volatile urinary components.

Fifty-one compounds were separated and identified from human urine with GC-MS, including several ketones, aldehydes, alcohols, pyrroles, and pyrazines. Pierce and Gearhart (1977) have since published a review article documenting chromatographic and mass spectrometric techniques utilized for the analysis of organic components of human urine.

The use of gas chromatography and/or mass spectrometry in entomological research has primarily been centered on the isolation and identification of female sex pheromones. Concerning ticks, Berger et al. (1971) were the first investigators to isolate the sex pheromone of the lone star tick, A. americanum, Gulf Coast tick, A. maculatum, and American dog tick, D. variabilis. Approximately 5000 partially engorged unmated female ticks of each species were homogenized several times and the extract separated by gas chromatography. The GC column effluent was then split between a flame ionization detector and a heated line that discharged part of the vapors into a stream of moving air directed over male ticks attached on a rabbit. A single component was found to cause characteristic behavioral responses in male ticks, similar to those observed prior to mating. The retention time of this compound was identical in all three species of ticks. Further experiments by Berger (1972), utilizing GC-MS procedures, identified this compound as 2,6-dichlorophenol (2,6-DCP).

Utilization of electrophysiological techniques in conjunction with gas chromatographic procedures was initially described by Ottoson and von Sydow (1964). These authors examined the odor intensities of GC-eluted volatile components of black currants by using an electro-olfactogram of a frog's olfactory membrane as a quantitative measure of odor potency. Car-3-ene and terpinen-4-ol were found to elicit the

highest relative electrophysiological responses. The combined usage of this system in entomological research was first described by Moorhouse et al. (1969), who electrophysiologically monitored the responses of male red bollworms, Diparopsis castanea Hampson, to female tip extracts as they eluted from a gas chromatograph. Although three components were found to consistently produce neurological responses in male moths, the chemical identity of these compounds was not determined. Since then, these techniques have been utilized for the isolation and identification of the female sex pheromones of several lepidopterous species (Nesbitt et al. 1975a, Nesbitt et al. 1975b, Nesbitt et al. 1977, Nesbitt et al. 1979). The possibility is then raised concerning the simultaneous usage of electrophysiology, gas chromatography, and mass spectrometry as an integrated procedure for the isolation, separation, and identification of other semiochemically important compounds (i.e., host attractants, stimulants, behavioral modifiers). A review of the literature, however, indicates that such an approach has never been documented in entomological research.

CHAPTER III

MATERIALS AND METHODS

Tick Rearing and Maintenance

Experiments were conducted on adult lone star ticks, Amblyomma americanum (Linnaeus), which had been reared on domestic rabbits and sheep at Oklahoma State University. Ticks were stored at 26 ± 0.5°C, and 87 ± 4% relative humidity under a 10L:14D photoperiod in 0.236 liter paper containers covered with Saron Wrap® held in place with a rubber band (Patrick and Hair 1975). Relative humidity was maintained by placing the containers in sealed Plexiglass® hydrating chambers containing a saturated K_2SO_4 solution (Winston and Bates 1960). These chambers were also designed to regulate ambient carbon dioxide concentration at 422 ± 65 ppm (Holscher et al. 1980).

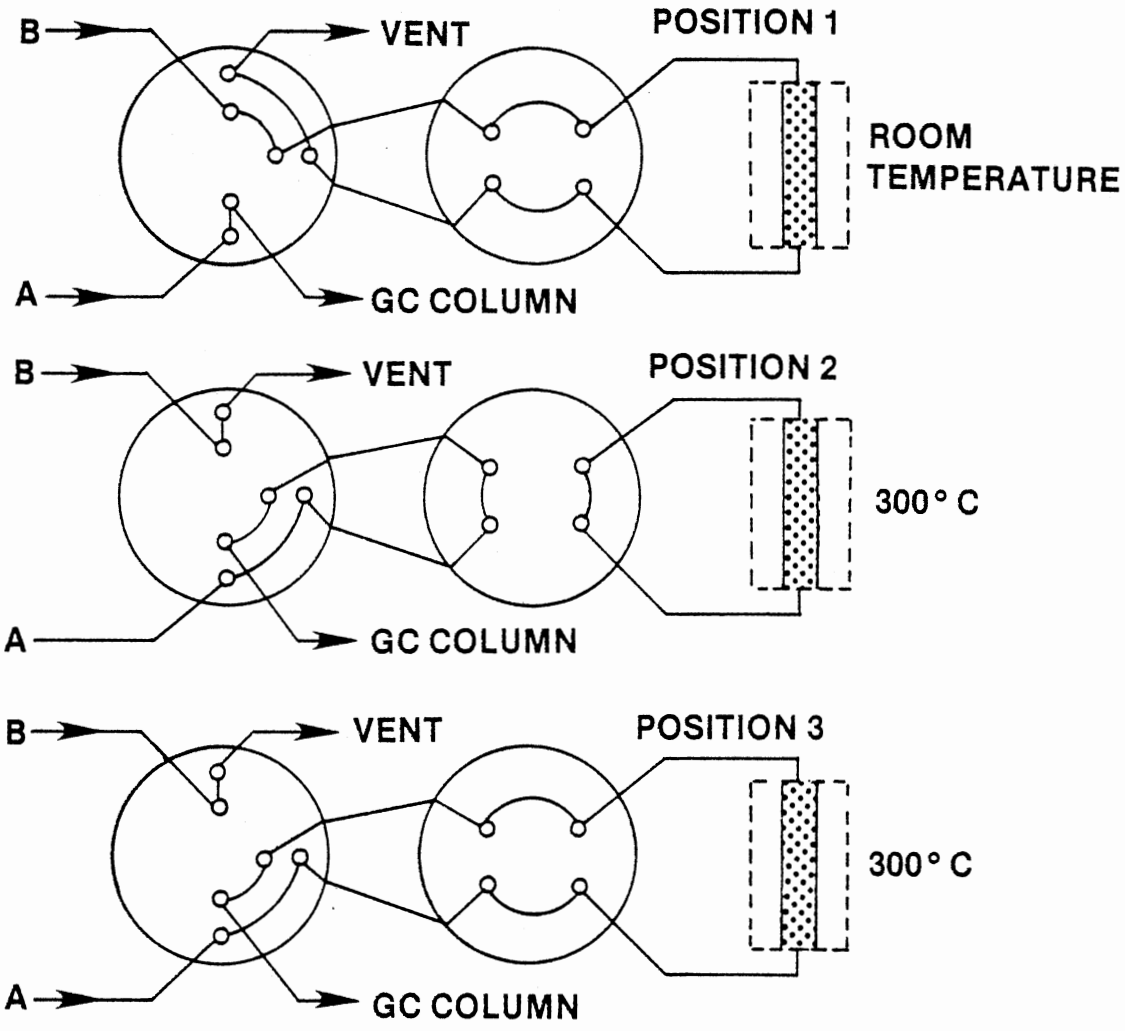
Sample Preparation

Four liters of urine was aseptically collected over a 5 day period from stanchioned female sheep at the Medical Entomology Laboratory. Prior to collection these animals were maintained on a standard feed mixture of ground corn, ground alfalfa, soy bean oil meal, and molasses (Ration No. SH019, Stillwater Milling Company, Stillwater, Oklahoma). All urine was pooled and stored at -10°C in stoppered glass containers until gas chromatographic analysis.

Collection and concentration of volatile urinary components was accomplished using a sparging system, with Tenax GC (poly-2,6-diphenyl-p-phenylene oxide) as the sorbing medium. A 100-ml aliquot of pooled sheep urine was placed in a 200-ml round-bottom glass sparging vessel which was then heated to 60°C with a heating mantle. A continuous stream of helium was passed over the urine for 2 hr at a flow rate of 100 ml/min using a Brooks® Model 8601 pressure regulator, and the head-space volatiles swept downstream through 0.0625 inch inner diameter (I.D.) stainless steel tubing and routed through a valve injection system (Figure 1). This system consisted of a Valco® Model V-6-HTa six-port sampling valve, and Valco® Model V-4-HTa four-port sampling valve which were encased in an aluminum heating block and thermally regulated at 175°C with a Hewlett Packard® Model 19121A heated zone temperature controller. The injection valves were then connected in circuit with a Tenax trap enclosed in a separate aluminum heating block. This trap consisted of a 3 x 0.125 inch I.D. stainless steel tube filled with 40 mg of 60/80 mesh Tenax GC (Applied Science Laboratories Inc.) and plugged at both ends with silanized glass wool.

Collection and concentration of volatile urinary components was accomplished with the Tenax trap at room temperature and the injection valves in position 1. After the sampling period, the Tenax trap was isolated (position 2) and the aluminum block surrounding the trap heated to 300°C with a 165 W heating cartridge regulated by a Valco® Model ITC-K10-999 temperature controller. The six-port and four-port valves were then switched, (position 3), under the control of a Hewlett Packard® Model 3385A automation system, and the thermally desorbed volatiles swept downstream and injected onto the column of a gas chromatograph.

Figure 1. Functions of Valve Injection System. Position 1. Sample Collection; Position 2. Sample Isolation and Heating; Position 3. Sample Desorbtion and Injection Onto GC Column. Dashed Line Represents Aluminum Heating Block Enclosing Tenax Trap. A and B Indicate Gas Inlets as Detailed in Figure 2.


B
VENT
POSITION 1
ROOM
TEMPERATURE
A
GC COLUMN
B
VENT
POSITION 2
300° C
A
GC COLUMN
B
VENT
POSITION 3
300° C
A
GC COLUMN

prior to injection, the GC column was cooled to 4°C with liquid carbon dioxide. This allowed the volatiles to condense as a slug at the front of the column preceeding GC separation. The dead volume of the injection system was minimized by using 0.01 inch I.D. stainless steel tubing for all connections. Between test runs, the Tenax trap was conditioned for 1 hr at 350°C under a constant helium purge.

Gas Chromatography

A Hewlett Packard® Model 7620A research chromatograph equipped with a flame ionization detector (FID) was used in this study. The syringe injection port was completely removed and the previously described valve injection system installed (Figure 2). Separation of volatile urinary components was accomplished on a 500 ft x 0.03 inch I.D. stainless steel open tubular column coated with methylsilicone oil (SF 96). Helium served as the carrier gas and was regulated at a flow rate of 5 ml/min with a Brooks® Model 8744 flow controller. A Hewlett Packard® Model 7660A multilevel programmer provided precise temperature programming at 3 different rates as well as isothermal operation. The temperature program used in this study was isothermal at 25°C for 30 min, increasing 0.5°C/min to 75°C, remaining isothermal at 75°C for 30 min, increasing 2°C/min to 125°C, remaining isothermal at 125°C for 10 min, increasing 8°C/min to 175°C, and remaining isothermal at 175°C for the remainder of the program. Other GC operating conditions are shown in Table I.

To compensate for the large dead volume which existed between the end of the GC column and the FID, a make up gas (helium) stream was installed at the end of the column. This was accomplished by installing a modified 1/4 to 1/16 inch low dead volume union which allowed the

Figure 2. Modified Gas Chromatograph and Injection System. A and B. Carrier Gas Inlets; 1. Flow Controller; 2. Pressure Regulator; 3. Sparging Vessel; 4. Sample Transfer; 5. Injection Valves in Heating Block; 6. Tenax Trap in Heating Block; 7. Injection Valve Outlet; 8. Injection Splitter; 9. GC Column; 10. Modified Low Dead Volume Union; 11. Effluent Splitter; 12. FID; 13. Make Up Gas Stream.

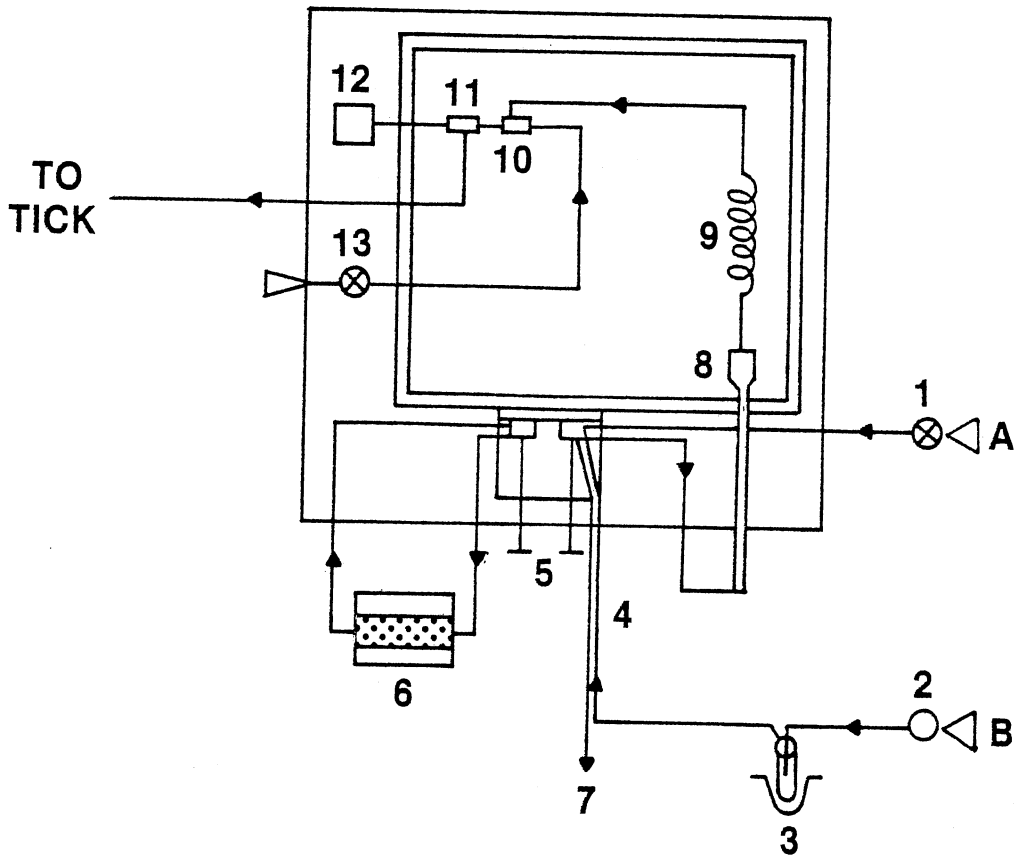

TO
TICK
12
11
10
13
9
8
1
A
5
4
6
2
B
7
3

TABLE I

GAS CHROMATOGRAPH OPERATING CONDITIONS

Gases:	Tank Settings	Approximate Flow Rate	Rotameter Settings
Hydrogen	15 psi	40 ml/min	FID 3.0
Air	26 psi	300 ml/min	FID 3.0
Helium (make up)	42 psi	60 ml/min	
Helium (carrier)	40 psi	5 ml/min	

Electrometer Settings:

Function	A
Supression	High
Range	10
Attenuation	64 (2^6)

FID Temperature: 275^{o}C

Chart Speed: 0.63 cm/min

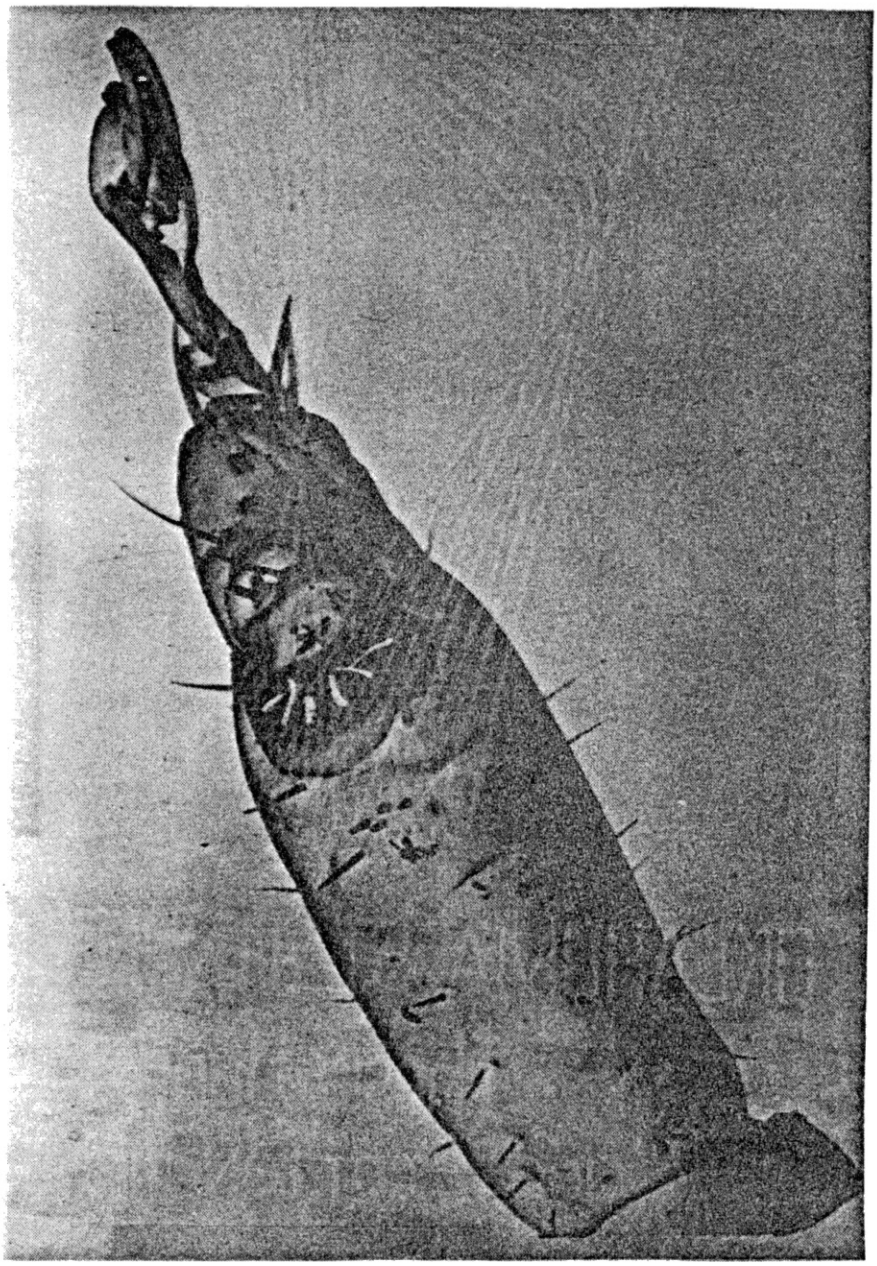
column effluent to be introduced into the side of the union and swept along at a higher flow rate. The make up gas flow rate was controlled with a Brooks® Model 8744 flow controller and insured optimum operating conditions for the FID. A Hewlett Packard® Model 19034A effluent splitter was then installed which allowed the column effluent to be split at a 1:1 ratio between the FID and a tick being monitored electrophysiologically. In essence, the tick was serving as an extension of the GC by functioning as a biologically selective odor detector. The split ratio between the two 'detectors' was determined by the length of a 0.01 inch I.D. tubing between the effluent splitter and the respective 'detectors'.

Electrophysiology

A copper-screen lined Faraday cage, like that used by Agee (1977), served as a holding area and provided an electrically grounded environment for tick preparation and testing. Ticks were immobilized ventral side up on double-sided tape and positioned on a 20 mm gas dispersion tube situated under a stereomicroscope. The dispersion tube was connected by 6 mm I.D. Tygon® tubing to a commercially prepared cylinder of 350 $\pm$ 18 ppm carbon dioxide (CO_2) in air (atmospheric). A constant flow of CO_2 was circulated through the dispersion tube and functioned to provide a stable gaseous environment during testing. The 0.0625 inch I.D. stainless steel GC column effluent line was then positioned in close proximity (ca. 2 mm) to Haller's organ (located on the tick's dorsal tarsal surface) (Figure 3).

Stainless steel microelectrodes were prepared according to Agee (1977), with a Throttlepack 501 transformer (Model Rectifier Corp.)

Figure 3. Haller's Organ Region of an Adult Female Lone Star Tick, Amblyomma americanum (Linnaeus).



serving as the electrode etcher. Sharpened electrodes were dipped in clear insulating solution and immediately placed in a vertical position which allowed insulation to form around the electrode shaft but not over the tip area. Using a pair of Brinkmann® Model H/I micromanipulators, one electrode was inserted into the tick body and served as the indifferent electrode. A similar recording electrode was micromanipulated through the corium between the metatarsus and tarsus just posterior to Haller's organ. Electrode leads were then connected to the input of a Grass® Model P15 differential AC preamplifier, and the amplified (1000X) and filtered nerve impulses displayed on a Tektronix® Model 5113 dual beam storage oscilloscope. A Model SA-10 solid state stereo amplifier and Model Solo-5 stereo speaker (Radio Shack Corp.) were also connected in circuit to the preamplifier and functioned to provide audible evidence of tick neural discharge activity. A schematic diagram of the combined GC-electrophysiological system is shown in Figure 4.

Tests were performed on 5 female and 5 male adult lone star ticks, A. americanum. Prior to GC-effluent stimulation, ticks were allowed a 30 min adjustment period in the closed Faraday cage. During this time a steady oscilloscope baseline was obtained, with few if any nerve impulses being produced. Following the initiation of GC-effluent stimulation, those peaks which produced a definite increase in tick neural discharge activity (Figure 5) were noted and their retention times recorded. Mean relative retention times were then calculated according to the method of McConnell and Novotny (1975), in which one peak is chosen as a reference and the retention times of the other peaks calculated relative to the reference peak.

Figure 4. Schematic Diagram of Combined Gas Chromatography-Electrophysiology System.

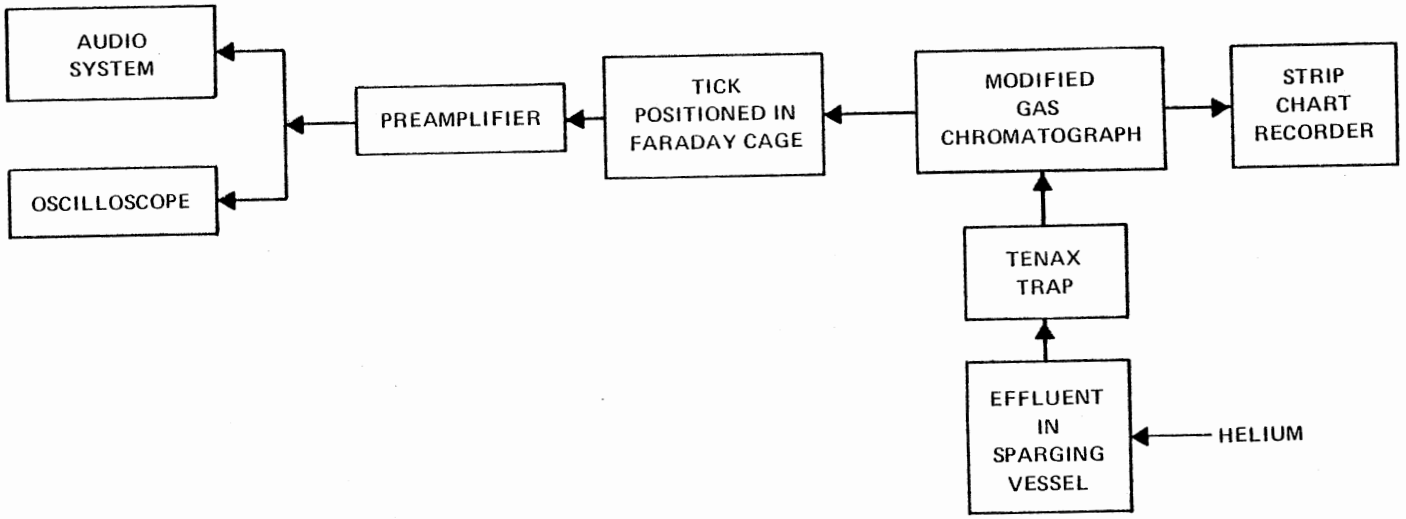
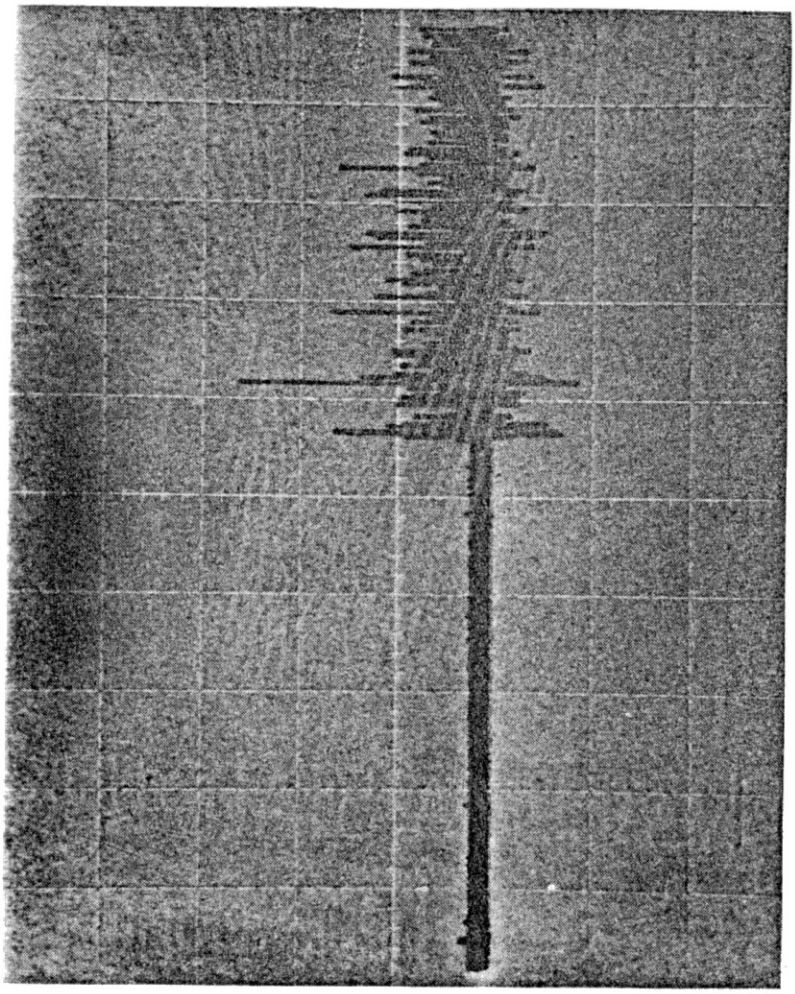

AUDIO SYSTEM
OSCILLOSCOPE
PREAMPLIFIER
TICK POSITIONED IN FARADAY CAGE
MODIFIED GAS CHROMATOGRAPH
STRIP CHART RECORDER
TENAX TRAP
EFFLUENT IN SPARGING VESSEL
HELIUM

Figure 5. Oscilloscope Tracing of Tick Neurological Activity During Exposure to Concentrated Volatile Components of Sheep Urine.



To check for contamination from extraneous sources, several blank runs were also conducted. This involved using the same previously described procedure, with the exception that no urine was placed in the sparging vessel.

Mass Spectrometry

Peaks eliciting electrophysiological responses were identified using gas chromatographic-mass spectrometric (GC-MS) techniques. Analysis were performed on the previously described GC system which was interfaced with a jet separator to a spectrEL Model 275-50 quadrupole mass spectrometer (Extranuclear Laboratories Inc.). Sample preparation and GC operating conditions were identical to those previously described with the exception that sample desorption and injection onto the GC column was accomplished according to the method of Priestly and Wilkes (1979). Electron impact mass spectra were recorded at an electron energy of 70 eV, with a separator temperature of 200^{o}C. The scan speed was 10 amu/sec for a mass range of 40-240 amu.

Preliminary identifications of the selected volatile components were based on comparisons with spectra of known compounds available. Final identifications were confirmed by comparing the mass spectra and retention times of the unknown compounds with those of authentic samples run under identical conditions.

CHAPTER IV

RESULTS AND DISCUSSION

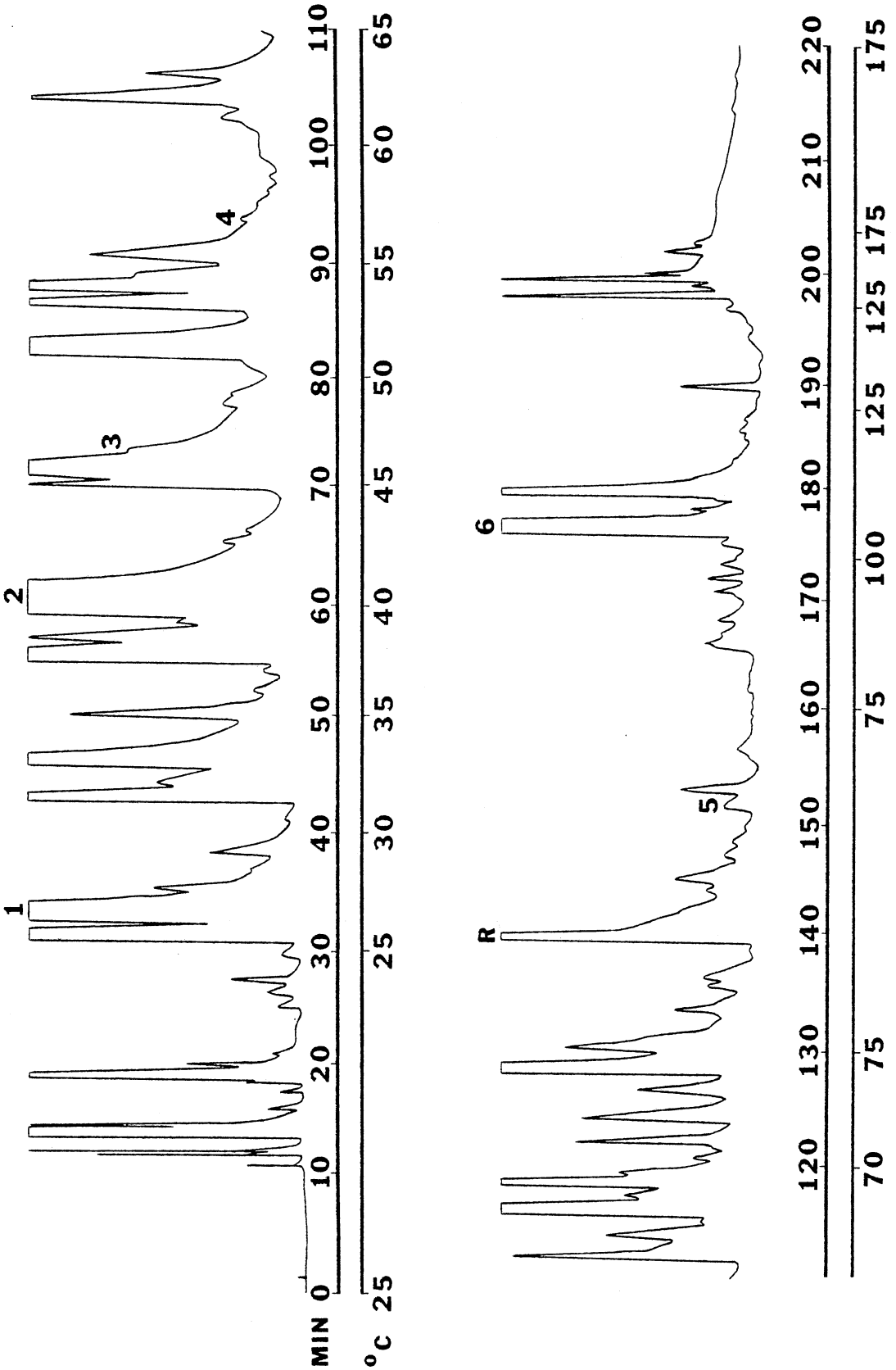
Gas Chromatography

A chromatogram of the concentrated volatile components of sheep urine is shown in Figure 6. Approximately 100-150 separate peaks of varying volatilities and concentrations eluted from the gas chromatograph (GC) during a total analysis time of 220 min. Good peak resolution and separation was obtained throughout the chromatogram, although some minor peak tailing was observed. These results indicate the reliability of the urinary sampling and modified GC systems, as well as the excellent trapping efficiency and retention volume of the adsorbent polymer Tenax.

Comparisons of individual chromatograms appeared quite similar, although some quantitative differences were evident. These variations were probably due to slight differences in urinary sampling periods, or the result of changes in the condition or performance of the GC column during individual analysis. The elution pattern and relative retention times of the peak components, however, remained relatively constant and attests to the reproducibility of the analysis system and the ability of the valve injection system to inject all of the components as a slug at the front of the GC column prior to separation.

Examination of blank chromatograms revealed only the presence of several small chromatographic peaks in the latter portion of the GC

Figure 6. Chromatogram of Concentrated Volatile Components of Sheep Urine. Numbered Peaks Indicate Components Which Elicited Electrophysiological Responses in Lone Star Ticks. R Denotes Reference Peak Used For Calculating Relative Retention Times.


1
2
3
4
5
6
R
MIN 0 10 20 30 40 50 60 70 80 90 100 110
°C 25 30 35 40 45 50 55 60 65
120 130 140 150 160 170 180 190 200 210 220
70 75 100 125 175

program. These constituents may have been the result of inherent contamination, memory effects from repeated use of the sampling system, or slight retention of urinary components on the Tenax trap from previous analysis.

Electrophysiology

As stated earlier, the temperature program used for chromatographic separation of the volatile urinary components produced a 220 min analysis period. This analysis time was quite lengthy, but none the less necessary in order to provide peak widths which were of adequate rise time for successful electrophysiological monitoring. Electrophysiological data collected from each chromatographic analysis showed that of the ca. 100-150 separate peaks which eluted from the GC column, only 6 components consistently elicited definite increases in tick neural discharge activity. These peaks are indicated in Figure 6. Also shown is a peak, denoted R, which was used for calculating the relative retention times of the numbered peaks. These relative retention times, along with the number and percentage of ticks responding to each numbered peak are shown in Table II.

Female ticks showed a high percentage or response to the selected volatile components, with only peak number 2 failing to elicit electrophysiological activity in all females tested. This is in contrast to the percentage of response observed in males, which ranged from zero in peak number 6, to 100% in peak number 2. These findings coincide with studies conducted by Holscher et al. (1980) involving CO_2 perception in A. americanum. Utilizing electrophysiological techniques, female ticks were found to consistently produce greater responses to regulated levels

TABLE II

SELECTED VOLATILE COMPONENTS OF SHEEP URINE WHICH ELICITED ELECTROPHYSIOLOGICAL RESPONSES IN LONE STAR TICKS

Peak Number	Mean Relative Retention Time	Number Responding[1] ♀	Number Responding[1] ♂	% Responding[1] ♀	% Responding[1] ♂
1	0.26 ± .004	5	1	100	20
2	0.47 ± .006	3	5	60	100
3	0.58 ± .009	5	4	100	80
4	0.73 ± .009	5	3	100	60
5	1.20 ± .009	5	2	100	40
6	1.36 ± .017	5	0	100	0

[1] Based on a total of 5 ♀ and 5 ♂ ticks.

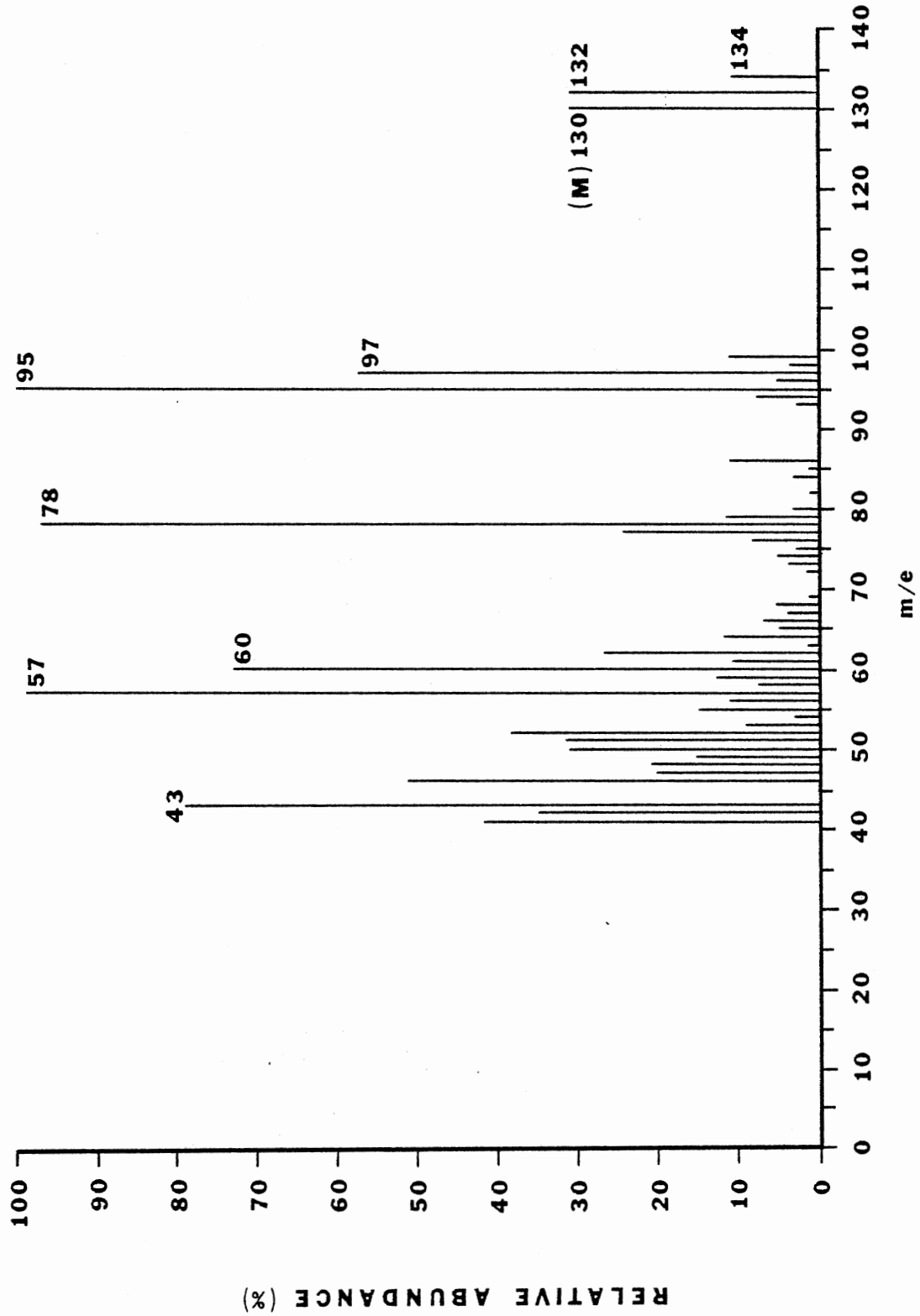
of CO_2 stimulation than males throughout all tests performed. These findings may indicate that female ticks possess a more complex or highly developed olfactory system, which could possibly be the result of innate differences in receptor potentials or number and types of olfactory binding sites. These results could also possibly be explained in terms of the differences in chemical composition or concentration of the selected volatile components. However, until a greater understanding of the neural and sensory physiology of ticks is obtained, any conclusions associated with these results must remain purely speculative.

Mass Spectrometry

A review of the literature indicates a lack of information regarding the isolation and identification of volatile components found in sheep urine. While this type of data is available concerning human urinary profiles (Zlatkis and Liebich 1971, Matsumoto et al. 1973, Zlatkis et al. 1973a, 1973c), obvious dietary and metabolic differences in these host species make any comparisons or correlations extremely dubious. Therefore, identification of the 6 selected volatile components must be based solely upon the interpretation of mass spectral data obtained.

The mass spectrum of peak number 1 is shown in Figure 7. The most obvious feature of this spectrum was the presence of an isotopic cluster at m/e 130, 132, and 134, which indicated an "A + 2" element (i.e., an element which has an isotope that is 2 mass units higher than its most abundant isotope). The characteristic pattern of this cluster, along with its isotopic ratios (m/e 132:130 = 0.99; m/e 134:130 = 0.35) strongly suggested the presence of a compound containing 3 chloride

Figure 7. Mass Spectrum of Volatile Urinary Peak Number 1.


RELATIVE ABUNDANCE (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
m/e
95
97
78
57
60
43
(M) 130
132
134

ions (Cl_3). The isotopic cluster at m/e 95 and 97 also indicated the presence of an "A + 2" element and could be accounted for by the loss of 1 Cl ion (M-35) from the molecular ion (M; m/e 130). The characteristic pattern and isotopic ratios (m/e 97:95 = 0.57; m/e 99:95 = 0.11) of this cluster also supported this conclusion. The major fragment ion at m/e 60 could then be explained by the loss of a second Cl ion (M-70) from the molecular ion. Assuming that the fragment ion at m/e 60 still contains 1 Cl ion, the remaining portion of this fragment would then have a molecular weight of 25. This could then be accounted for by the presence of 2 carbon and 1 hydrogen ions (C_2H).

The above data gave strong evidence supporting a compound having the empirical formula C_2HCl_3. A review of the spectra of known compounds available, along with certain physical characteristics of the unknown compound (i.e., its relatively high volatility), suggested the possibility of peak number 1 being 1,1,2-trichloroethylene (TCE). Although chlorinated organic compounds are rare in biological systems, recent evidence has indicated an increasing prevalence of TCE which made this possibility feasible (U. S. EPA 1980).

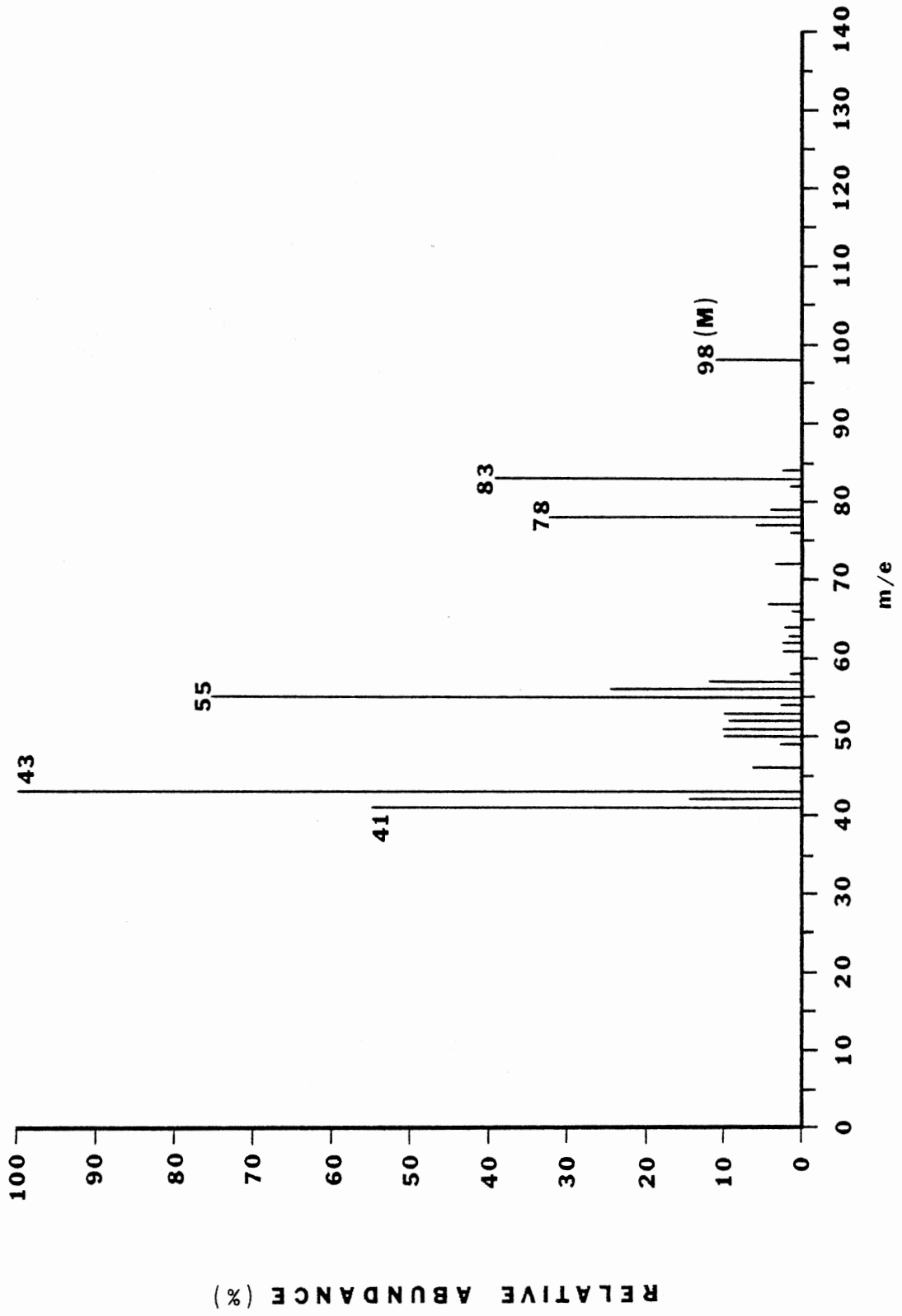
An authentic sample of the headspace volatiles of TCE gave a retention time and fragmentation pattern identical to that of the unknown compound. It should be noted that in the unknown spectrum, major fragment ions were observed at m/e 78 and 57 which cannot be adequately accounted for and are usually not present at these high abundances in the spectrum of TCE. These fragments were, however, observed in both the unknown and TCE spectra run under our testing regimes. An examination of the background activity obtained from our mass spectrometer indicated a relatively high abundance at these m/e, which may possibly

account for these discrepancies.

In 1972, Berger isolated and identified the female sex pheromone of three ixodid tick species, including A. americanum, as 2,6-dichlorophenol. At the time of this discovery doubt was raised as to the attractiveness of this component, as chlorinated compounds had never been previously associated with tick olfactory perception or sensory physiology. However, further studies by Burris (1974) on possible candidate chemical attractants for A. americanum, also indicated good attractive responses to several chlorinated compounds. These findings, in conjunction with our results, suggest that A. americanum may possess specific chloride-sensitive olfactory receptors or binding sites which may possibly be utilized in host-orienting activity or other unspecified behavior. More research, however, will be required to establish the validity of this assumption.

The mass spectrum of peak number 2 is shown in Figure 8. The molecular ion (M) was observed at m/e 98, with a corresponding base peak at m/e 43. The 41, 55, and 83 ion series strongly suggested the possibility of an unsaturated aliphatic hydrocarbon, or a cycloalkane. The fragment ion at m/e 83 (M-15) could be the result of the loss of a methyl group (CH_3) from the molecular ion. The m/e 55 (M-43) fragment could then be explained by the corresponding loss of a carbonyl group (C=O) in conjunction with the CH_3 group from the molecular ion. The loss of this CH_3CO^+ group would then account for the base peak at m/e 43. The fragment ion at 43 (M-55) also indicates that the other group attached to the carbonyl must have the formula C_4H_7, therefore suggesting an alkene. The loss of a stable neutral H_2 molecule would then account for the fragment ion at m/e 41. A large fragment at m/e 78 was

Figure 8. Mass Spectrum of Volatile Urinary Peak Number 2.


RELATIVE ABUNDANCE (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
m/e
43
41
55
83
78
98 (M)

once again observed and attributed to the high background activity at this m/e which was inherent in our system.

The above data gave strong evidence supporting a methyl ketone having the empirical formula $C_6H_{10}O$. A review of the spectra of known compounds available suggested the possibility of peak number 2 being 4-methyl-3-pentene-2-one (mesityl oxide), which has previously been identified in human urinary profiles (Zlatkis and Liebich 1971, Matsumoto et al. 1973, Zlatkis et al. 1973a, 1973c).

An authentic sample of the headspace volatiles of mesityl oxide gave a fragmentation pattern similar to that of the unknown compound, but eluted from the GC column at a different retention time. Authentic samples of methylcyclohexane and methylcyclopentanone were also examined as possibilities for peak number 2. Both of these compounds, just like mesityl oxide, have been previously identified in human urinary profiles and have reference spectra similar to that of the unknown compound. Once again, however, even though the authentic spectra of these compounds superficially resembled that of peak number 2, their retention times differed. The exact identity of peak number 2, therefore, remains unknown although the empirical formula $C_6H_{10}O$ appears to be valid.

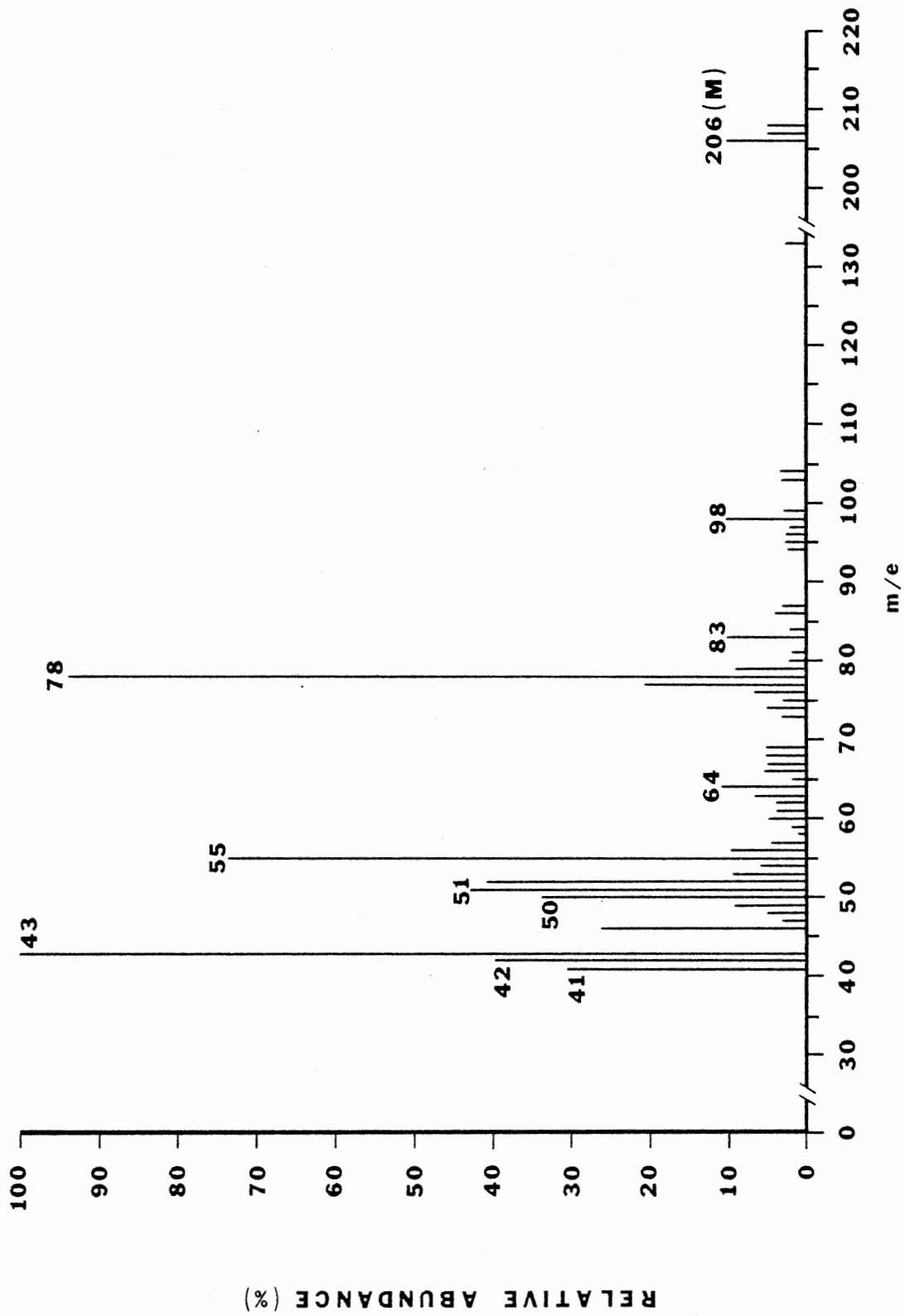
Methyl ketones have been shown to impart a communicative function in several insect species, which includes their use as alarm and trail-marking substances (Cavill and Hinterberg 1960, Duffield et al. 1977), and aggregation pheromones (Rudinsky et al. 1974, Ryker et al. 1979). Although the significance of these compounds in tick olfaction has not been investigated or reported, our findings indicate their possible involvement as specific olfactory stimuli associated with host-orienting behavior. Whether or not this assumption is valid and extends to a

wide range of methyl ketones or only to those specific compounds having the empirical formula $C_6H_{10}O$, however, cannot be determined by the data presented here and remains open to future investigations.

The mass spectrum of peak number 3 is shown in Figure 9. The molecular ion (M) was observed at m/e 206, with M+1 and M+2 fragments occurring at m/e 207 and 208. A prominent base peak was observed at m/e 43, along with other major fragment ions at m/e 41, 42, 50, 51, 52, 55, 64, 78, 83, and 98. A review of the spectra of known compounds available indicated no possible matches for this spectrum. While the spectra of several compounds having a molecular ion at m/e 206 were recorded, none had fragmentation patterns similar to that of peak number 3.

One possible explanation for the lack of a matching compound could be that the spectrum recorded for peak number 3 may actually be the result of more than one component (i.e., mixed spectra). This phenomenon has been described as a problem frequently encountered in the analysis of volatile components of odor isolates (Kolar 1972). If this assumption of mixed spectra is valid, the specific component responsible for the elicitation of electrophysiological responses cannot be accurately accounted for. However, the major portion of this spectrum, consisting of fragment ions at m/e 41, 43, 55, 83, and 98, superficially resembles the fragmentation pattern observed in peak number 2. This may indicate that one of the components in the spectrum could be another methyl ketone having the empirical formula $C_6H_{10}O$. The formation of these major fragment ions could then be explained in a manner similar to that previously described. It should also be noted that the highest percentages of male response were elicited from peaks number 2 and 3,

Figure 9. Mass Spectrum of Volatile Urinary Peak Number 3.


RELATIVE ABUNDANCE (%)
100
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70
60
50
40
30
20
10
0
0
30
40
50
60
70
80
90
100
110
120
130
200
210
220
m/e
206(M)
98
83
64
78
55
51
50
43
42
41

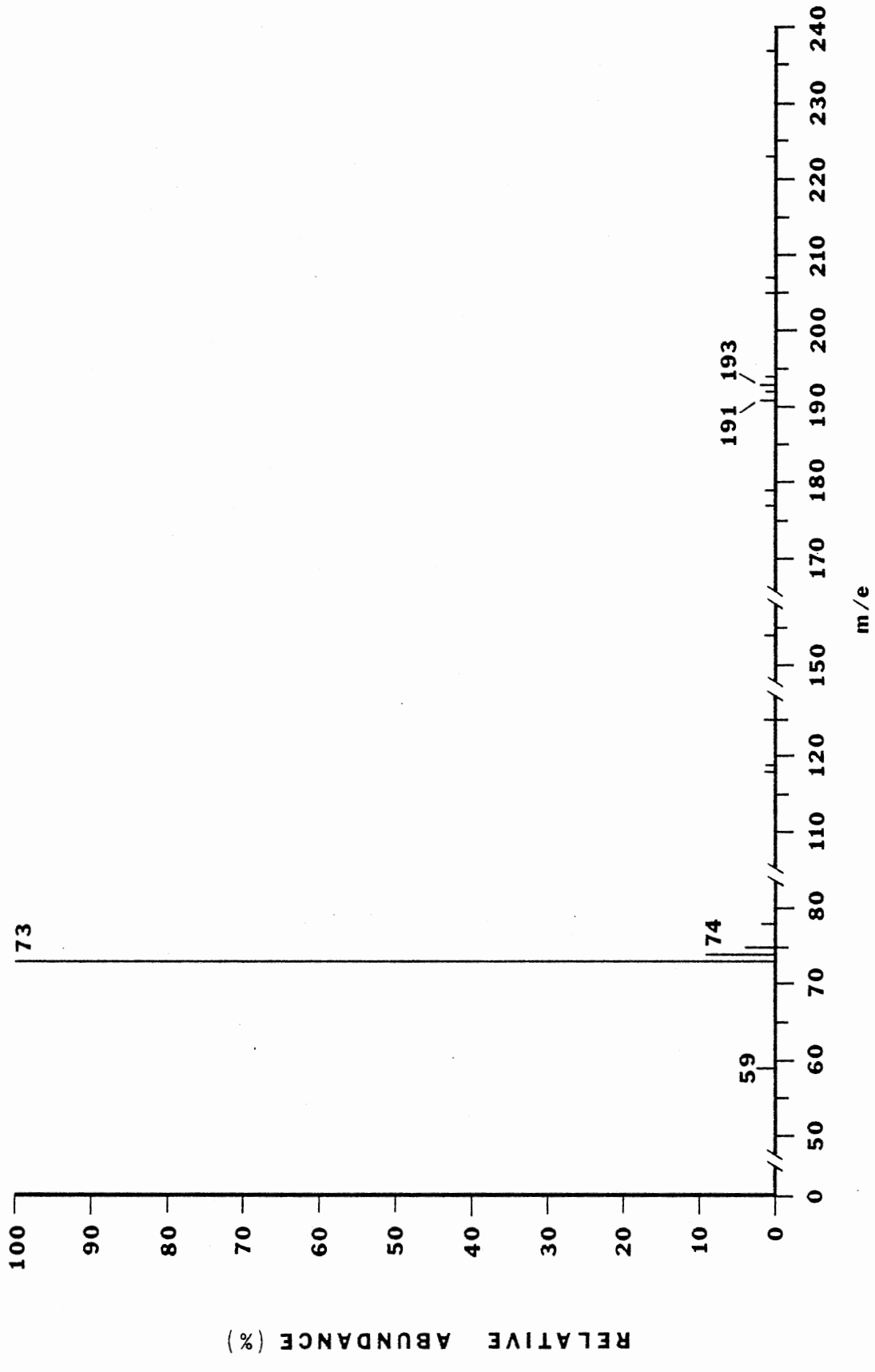
which adds further support to the possible presence of another methyl ketone component in the spectrum of peak number 3. However, until a better separation of this chromatographic region can be obtained, possibly through the use of a different temperature program or GC column, any conclusions concerning mixed spectra must be viewed with caution. The possibility still remains that the spectrum obtained from peak number 3 is in fact real, and represents a component which has not been previously isolated or identified.

Mass spectral data for peaks number 4 and 5 are unavailable. The extremely small concentrations of these peaks which eluted from the GC column were not sufficient to allow successful mass spectrometric analysis. Attempts to increase these concentrations by increasing the urinary sampling period were also unsuccessful.

The lack of identities for peaks number 4 and 5 does not diminish from their importance or significance. The fact that these peaks were able to elicit electrophysiological responses even when found in such extremely small abundances atests to the high level of sensitivity of the tick olfactory system. These results also point out the selectivity which may be involved in the perception of host-emitted olfactory stimuli. Even though a large number of volatile components may contribute to the overall odor emitted by a perspective host, the mere presence of specific components, regardless of concentration, may be required to initiate and influence host-orienting activity.

The mass spectrum of peak number 6 is shown in Figure 10. The obvious feature of this spectrum was the extremely abundant fragment ion at m/e 73, which also represented the base peak. The remainder of the spectrum was comprised of extremely small fragment ions, the most

Figure 10. Mass Spectrum of Volatile Urinary Peak Number 6.


RELATIVE ABUNDANCE (%)
100
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60
50
40
30
20
10
0
0
50
60
70
80
110
120
150
170
180
190
200
210
220
230
240
m/e
73
74
59
191
193

notable of which occurred at m/e 59, 74, 75, 191, and 193. No prominent molecular ion (M) was observed. A review of the literature indicated that this type of fragmentation pattern is very characteristic of siloxane compounds. These types of compounds, however, are extremely rare in biological systems and suggested the possibility that the unknown compound was not a urinary component, but the direct result of inherent contamination within our testing system. This theory was further supported by examination of blank chromatograms which indicated that a peak characteristically occurring in the latter portion of these programs had a retention time similar to that of peak number 6. It should also be noted that electrophysiological responses were elicited from this peak in female ticks tested during blank chromatograms.

One possible explanation for the presence of this peak could be the slight breakdown of the methylsilicone oil (SF 96) which was used as the stationary phase in the GC column. This particular compound is a high molecular weight methylpolysiloxane having the empirical formula $(CH_3)_3SiO\left[Si(CH_3)_2O\right]_nSi(CH_3)_3$. Breakdown of this polymer could then conceivably lead to the formation of fragment ions at m/e 59 (CH_3SiO^+), m/e 73 ($C_3H_9Si^+$), m/e 74 ($C_2H_6SiO^+$), and m/e 75 ($C_2H_7SiO^+$). Specific rearrangement reactions could then be responsible for the fragment ions at m/e 191 ($C_5H_{15}Si_3O_2^+$), and m/e 193 ($C_5H_{17}Si_3O_2^+$).

Although the majority of the GC carrier gas is effectively removed through the use of an interfacing separator, small amounts of the stationary phase from the GC column are swept into the mass spectrometer (Elliot 1972). Accordingly, the mass spectrum of this material should exhibit a fragmentation pattern characteristic of that particular phase. A review of the spectra of known compounds available indicated that the

fragmentation pattern of this specific siloxane polymer, SF 96, has not been recorded. A somewhat similar spectrum, however, has been observed for the dimethylpolysiloxane stationary phase, OV-1, and for a decamethylcyclopentasiloxane having the empirical formula $C_{10}H_{30}O_5Si_5$.

The discovery that peak number 6 is not a naturally occurring component of the urinary profile casts doubt as to its specific involvement in tick host-orienting behavior. A significant amount of information regarding tick olfaction, however, may be gained from this finding. Peaks number 2, 6, and possibly 3 all contain oxygen as an integral part of their chemical composition. This suggests that A. americanum may possess specific oxygen-sensitive receptors or binding sites which could possibly function in host perception. A similar rationale has been purposed by Washio and Nishino (1976) to explain the food-locating behavior in cockroaches, but has never been postulated concerning this activity in ticks. These receptors could also conceivably be responsible for the perception of CO_2, which has generally been associated with tick host-orienting behavior. This theory, however, cannot explain the lack of response observed in male ticks to the exygenated compound identified in peak number 6. The spatial configuration of this compound may account for this discrepancy, as could any inhibitory effect which might be imparted by the silicone portion of this component. A closer evaluation of peaks number 2 and 3 also reveals that the oxygen portion of these compounds is incorporated as part of a carbonyl (C=O) functional group. This may indicate that male ticks are only perceptive to oxygen when it is contained within these specific functional groups. The perception of CO_2 would still be accounted for under these conditions. It must be emphasized, however, that these assumptions are

purely hypothetical. To base any definite conclusions concerning the presence or types of tick olfactory receptors solely on the data presented here would be imprudent. Valid confirmation can only come through future investigations.

CHAPTER V

SUMMARY AND CONCLUSUIONS

This investigation was designed to determine if potential tick host-orienting olfactory stimulants or attractants are present in the volatile organic components emitted from host body effluents. Sheep urine was used as the effluent of choice and systematically analyzed using headspace collection and concentration techniques followed by simultaneous gas chromatographic (GC) separation and electrophysiological monitoring. Those components which elicited definite increases in tick neural discharge activity were then analyzed and identified using mass spectrometric (MS) techniques.

The urinary sampling and GC system as a whole provided efficient collection, maximum concentration, and excellent separation of the volatile organic components. Each chromatographic analysis produced ca. 100-150 measurable peaks of varying volatilities and concentrations. Comparisons of individual chromatograms were quite similar and demonstrated the excellent reproducibility of the analysis system.

Electrophysiological data collected from each chromatographic analysis showed that only 6 peaks consistently elicited definite increases in tick neurological activity. Differences were observed in the number of ticks responding to each of the selected peaks, with females generally being more responsive.

Mass spectral identities of the selected peaks included 1,1,2-

trichloroethylene, a methyl ketone having the empirical formula $C_6H_{10}O$, an as yet unknown component, and a methylpolysiloxane resulting from column bleed of the GC stationary phase. The remaining 2 peaks were not identifiable due to their extremely low abundances.

These findings point out the extreme sensitivity and selectivity of the tick olfactory system, and indicate the possible presence of chemically specific olfactory receptors or binding sites. The possible involvement of these specifically identified volatile components in tick host-orienting activity or other unspecified behavior is also indicated. The biological relevance of these findings, however, remains to be demonstrated. As in all electrophysiological investigations of this type, behavioral tests must be conducted and results correlated before any assumptions can be confirmed.

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